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(54) Title: MULTI-UNIT RIBOZYME INHIBITION OF ONCOGENE EXPRESSION

(57) Abstract

A novel multi-unit ribozyme is provided which is capable of selectively cleaving the mRNA transcript of a hybrid oncogene resulting from a chromosomal translocation. Specialized delivery vehicles such as liposomes, and growth factor conjugates for cellular uptake by receptor-mediated endocytosis, are also described. The multi-unit ribozyme is used for the treatment of neoplasms characterized by expression of the oncogene. In one embodiment, a multi-unit ribozyme is provided which cleaves the mRNA transcript of Philadelphia chromosome-positive cells, thereby blocking production of the tumorigenic $p210^{bcr-abl}$ fusion protein.

MULTI-UNIT RIBOZYME INHIBITION OF
ONCOGENE EXPRESSION

Field of the Invention

5   The invention relates to the inhibition of oncogene expression by ribozymes complementary to oncogene mRNA transcripts. The invention further relates to the therapeutic use of such ribozymes in the treatment of neoplastic diseases. In one embodiment, the invention relates to the treatment of
10   leukemias characterized by the Philadelphia chromosome translocation.

Reference to Government Grant

Background of the Invention

        Chronic myelogenous leukemia (CML) accounts for 20% of
20   all cases of leukemia and carries a death rate of 1.5 per 100,000. population (Lichtman, Chronic Myelogenous Leukemia and Related Disorders in Hematology, Williams (ed.), 4th Ed., McGraw Hill, New York, N.Y. (1990)). In 1960, Nowell and Hungerford discovered that the Philadelphia chromosome (Ph+)
25   was consistently associated with CML (reviewed in Rosson and Reddy, Mutation Res., 195, 231-243 (1988)). Molecular studies have demonstrated that during the formation of the Philadel-phia chromosome, a portion of the c-abl gene is translocated from chromosome 9q34 to chromosome 22q11, resulting in the
30   formation of a chimeric gene consisting of sequences derived from the BCR and ABL loci (Heisterkamp et al., Nature, 306, 239 (1983); Heisterkamp et al., Nature, 315, 758 (1985)). This translocation is detectable in over 95% of patients with

- 2 -

CML. A significant portion of acute lymphocytic leukemia (ALL) patients also carry the Ph¹ chromosome.

The fusion gene, named bcr-abl, transcribes a chimeric mRNA of 8.5 kb which is translated to a

5   p210$^{bcr-abl}$ fusion protein with altered tyrosine kinase activity (Konopka and Witte, Mol. Cell Biol., 5, 3116-3123 (1985); Kurzrock et al., N. Engl. J. Med., 319, 990 (1988)). The p210$^{bcr-abl}$ protein has been shown to transform myeloid precursor cells in vitro (McLaughlin et al., Proc. Natl. Acad. Sci.

10   USA, 84, 8558 (1987)). In addition, infecting murine bone marrow stem cells with a retrovirus containing the bcr-abl gene produces a disorder similar to human CML in mice (Daley et al., Science, 247, 824 (1990); Voncken et al., Blood, 79, 1029-1036 (1992)).

15   It has been shown using antisense molecules that interrupting the bcr-abl transforming signal can inhibit the cell growth of isolated blast cells from CML patients (Szczylik et al., Science, 253, 562 (1991); WO 92/22303 (1992)).

20   In the past decade, the use of antisense nucleic acid sequences to block the function of a mRNA has been developed as a strategy to inhibit viral and malignant diseases (Weintraub, Sci. Amer. 262, 34-40 (1990)). More recently, it has been shown that specific RNA sequences have catalytic

25   activity (Kruger et al., Cell 81, 147-157 (1982)). These molecules are termed "ribozymes". Several different catalytic sequences have been described with a common need for divalent cations, which cause nonhydrolytic transesterification of specific RNA target regions (Symons, Annu. Rev. Biochem. 61,

30   641-671 (1992)). These catalytic sequences have been modified to create antisense molecules which bind to and cleave specific target RNA molecules (Zaug et al., Nature, 324, 4229-4233 (1986); Uhlenbeck, Nature 328, 596-600 (1987)).

Snyder et al., Blood 82, 600-605 (1993) have shown that

35   liposome vectors containing a single-unit DNA-RNA hybrid ribozyme can inhibit bcr-abl gene expression and cell growth in a Ph-positive cell line. Other reports of ribozyme-mediated inhibition of bcr-abl gene expression include WO 93/03141 (1993), and WO 92/00080 (1992) and the corresponding

- 3 -

Reddy et al., U.S. Patent 5,246,921. The "hammerhead" ribozyme has been developed into a targeted ribozyme, employing a catalytic "hammerhead" domain and flanking oligonucleotides that specifically bind to target sequences (Walbot, Nature 334, 585-591 (1988)). Cleavage occurs 3' to a GUX triplet where X can be C, U, or A (Guerrier-Takada et al., Cell 35, 848-852 (1983); Cech & Bass, Annu. Rev. Biochem. 55, 599-629 (1986); Symons, Trends Biochem Sci. 14, 445-50 (1989)). Any GUX sequence can be targeted by the appropriate design of the flanking oligonucleotide sequence.

There are no conventional therapies including chemotherapy, ^{32}P-treatment and splenic irradiation, that have resulted in cures in CML (Lichtman, Chronic Myelogenous Leukemia and Related Disorders in Hematology, W. J. Williams, Ed., 4th Edition, McGraw Hill, New York, N.Y. (1990)). Five to 15% of patients receiving alpha-interferon therapy may suppress the expression of the Ph-positive clone in CML (Talpaz et al., N. Engl. J. Med. 314, 1865-1859 (1986). However, the ability of interferon to cure CML is not proven. Allogeneic bone marrow transplant (BMT), using HLA identical siblings, following myeloablative chemo-radiotherapy is curative in up to 85% of carefully selected patients in chronic phase CML (Goldman et al., N. Engl. J. Med. 314, 202-207 (1986); Thomas et al., Amer. Intern. Med.104, 155-166 (1986); Marks et al., Br. J. Haematol. 81, 383-390 (1992)). In CML patients transplanted with an identical twin following high dose myeloablative therapy, the cure rate is 50%. This difference is due to a graft-versus-leukemia (GVL) effect and is enhanced by the presence of chronic graft versus host disease (GVHD). T-cell depletion of donor marrow reduces the GVL effect, the incidence of GVHD, as well as the cure rate (Gale et al., Bone Marrow Transplantation 9, 83-85 (1992)). Collectively, these data show that high dose therapy given early in the course of CML is potentially curative in 50% of patients. The additional curative effects of allogeneic BMT are due to a GVL effect.

Unfortunately, less than 30% of CML patients will have a normal allogeneic HLA matched donor. Current use of matched unrelated donors has resulted in high mortality due to GVHD

- 4 -

and infections (Marks et al., Ann. Int. Med. 119, 207-214 (1993)). Thus, the development of an autologous marrow transplant program using Ph-negative stem cells, would provide an alternative for patients without other curative options. An autologous BMT would avoid GVHD and should be curative in up to 50% of patients, provided the marrow is purged free of CML stem cells. In preparation for autologous marrow infusion, marrow cells are harvested from the affected individual, are "purged" of leukemia cells by chemical agents, and returned to the patient following extensive chemotherapy or total body radiation.

Thus far, autologous BMT has not been successful in CML because Ph-positive stem cells are invariably reinfused into the patient (Reiffers et al., British Haematology 77, 339-345 (1991)). In an attempt to provide Ph-negative stem cells for autologous BMT, chemotherapeutic purging (Silvestri et al., Int. J. Cell Cloning 9, 474-490 (1990)), interferon purging (McGlave et al., Bone Marrow Transplantation 6, 115-120 (1990)), long term bone marrow cultures (Barnett et al., Bone Marrow Transplantation 4, 345-351 (1989)), and CD34 stem cell selection (Verfaillie et al., Blood 79, 1008-1010 (1992)) have all been attempted and may result in transient Ph-negative hematopoiesis. Using antisense molecules, Szczylik et al., Science, 253, 562 (1991) and WO 92/22303 (1992) inhibited the cell growth of isolated blasts from CML patients.

While the presently known bcr-abl ribozymes (Snyder et al., Blood 82, 600-605 (1993) and WO 93/03141 (1993); Reddy et al., WO 92/00080 and U.S. Patent 5,246,921) are promising, there is a need for bcr-abl ribozymes with increased catalytic potential for oncogenic bcr-abl mRNA, without causing substantial cleavage of normal c-abl and bcr transcripts. There is also a need for bcr-abl ribozyme vectors having improved uptake.

Summary of the Invention

A synthetic RNA molecule useful for the treatment of a neoplasm characterized by the presence of a hybrid oncogene resulting from a chromosomal translocation is provided comprising

- 5 -

(a) a first ribozyme subunit comprising

(i) a first flanking segment having a nucleotide sequence complementary to the nucleotide sequence of a portion of the oncogene mRNA transcript substantially 5' of the oncogene translocation junction and being hybridizable to that mRNA portion;

(ii) a second flanking segment having a nucleotide sequence complementary to the nucleotide sequence of a portion of the oncogene mRNA transcript substantially 3' of the bcr-abl translocation junction and being hybridizable to that mRNA portion;

(iii) a catalytically active segment disposed between the first and second flanking segments comprising a ribozyme capable of cleaving the oncogene mRNA at or near the translocation junction; and

(b) two or more additional ribozyme subunits, each subunit comprising a catalytically active segment comprising a ribozyme capable of cleaving the oncogene mRNA and flanking segments complementary to the bcr-abl mRNA transcript and hybridizable thereto.

According to one embodiment of the invention, a synthetic RNA molecule is provided for the treatment of leukemia. For example, where the oncogene is bcr-abl, a synthetic RNA molecule targeting the bcr-abl mRNA is provided, which may be used for the treatment of Ph-positive leukemias.

According to another embodiment, a recombinant vector is provided comprising DNA which when expressed will provide such a synthetic RNA molecule.

In yet another embodiment, the invention is a pharmaceutical composition comprising the aforesaid synthetic RNA molecule and a pharmaceutically acceptable carrier. The carrier may comprise, for example, a liposome.

In a further embodiment, the invention takes the form of a conjugate comprising the synthetic RNA molecule linked to a cell surface ligand-binding molecule, for improved cellular uptake.

- 6 -

A therapeutic method for the treatment of a patient afflicted with a neoplasm characterized by the presence of a hybrid oncogene resulting from a chromosomal translocation is provided. The method comprises contacting cells of the patient with an effective amount of the synthetic RNA molecule for a time sufficient to cleave the oncogene mRNA and inhibit the expression of that gene. The method may be carried out _in vivo_ or _in vitro_. According to an _in vitro_ method comprising a bone marrow purging procedure, bone marrow cells aspirated from a leukemia patient, e.g., a Ph-positive leukemia patient, are treated with a synthetic RNA molecule of the invention to purge leukemic cells. The purged marrow cells are then returned to the body of the afflicted individual.

The proliferation of cells characterized by the presence of a hybrid oncogene resulting from a chromosomal translocation may be inhibited by introducing into such cells an artificially-constructed gene which, upon transcription in said cell, produces the synthetic RNA molecule of the invention, which cleaves the oncogene mRNA and thereby inhibits proliferation of the cells. The artificially-constructed gene may be introduced into the susceptible cells by, for example, transfection, by a transducing viral vector or by microinjection.

Description of the Figures

Fig. 1 is a schematic of a triple-unit ·ribozyme according to the invention (SEQ ID NO:1), shown in hybridizing relationship with the translocation junction portion of the target complementary bcr-abl mRNA transcript. Restriction sites for assisting in the cloning of the molecule, or in the cloning of subunits thereof, are indicated. The translocation junction is also indicated.

Fig. 2 is an autoradiogram of the ribozyme-mediated cleavage of a target bcr-abl mRNA synthesized from a plasmid vector containing a 499 bp segment of the bcr-abl chimeric gene. Each lane shows the cleavage products resulting from the reaction of single-, double-or triple-unit ribozymes targeting sites #1, #2 and/or #3 of the bcr-abl mRNA shown

in Fig. 1:   Lane A - single-unit ribozyme (SEQ ID NO:7)
targeting site #2; lane B - single-unit ribozyme (SEQ ID NO:2)
targeting site #1; lane C - single-unit ribozyme (SEQ ID NO:3)
targeting site #2; lane D - single-unit ribozyme (SEQ ID NO:4)

5   targeting site #3; lane E - double-unit ribozyme (SEQ ID NO:5)
targeting sites #1,2; lane F - double-unit ribozyme (SEQ ID
NO:6) targeting sites #2,3; lane G - triple-unit ribozyme (SEQ
ID NO:1) targeting sites #1,2,3; and lane S - control
containing substrate without ribozymes.

10   Figs. 3A and 3B comprise autoradiograms of ribozyme-
mediated cleavage of c-abl (Fig. 3A) and bcr (Fig. 3B) mRNA
which include the breakpoint region. Fig. 3A, lane S shows
the cleavage of c-abl control mRNA (no ribozymes), in
comparison to cleavage with ribozymes D and G. Fig. 3B shows

15   the cleavage of bcr control mRNA (no ribozymes), in comparison
to cleavage with ribozymes B and G.

          Figs. 4A, 4B and 4C comprise autoradiograms of
transfection by a triple-unit ribozyme with different delivery
vehicles. Fig. 4A compares K562 cell transfection with naked

20   ribozyme (lane 1) versus transfection with ribozyme in
liposome vector. Figure 4B and 4C compare 32D cell
transfection using 1 μg of ribozyme with increasing quantities
of folate-polylysine (Fig. 4B) or lipofectin (Fig. 4C) as a
vehicle.

25   Fig. 5 is an autoradiogram from sequential transfections
of triple-unit ribozymes via folate-mediated uptake in 32D
cells. Total cellular RNA was isolated 3 hour after
transfection. The lanes marked "3", "24" and "48" represent,
respectively, RNA from cells transfected at time zero with

30   ^{32}P-labeled ribozymes, RNA from cells transfected at time zero
with unlabeled ribozymes and at 24 hours with ^{32}P-labeled
ribozymes, and RNA from cells transfected at time zero with
unlabeled ribozymes and at 48 hours with ^{32}P-labeled ribozymes.

          Fig. 6 is an autoradiogram of a Southern blot of RT-PCR-

35   amplified bcr-abl mRNA from transformed 32D cells transfected
with vectors containing no ribozymes (Control) or triple-unit
ribozymes in folate-polylysine (Folate transfections) or
liposome vectors (Liposome transfections).

- 8 -

Detailed Description of the Invention

The invention provides a synthetic RNA molecule which acts as a multi-unit ribozyme. The multi-unit ribozyme more efficiently cleaves target oncogene mRNA than single unit ribozymes alone or in combinations. The inventive ribozyme is specific for the hybrid oncogene, since it only poorly cleaves mRNA transcripts of the parent proto-oncogenes whose translocation results in the hybrid oncogene. Thus, only cells containing the chromosomal translocation resulting in the expression of the oncogene are affected.

Without limiting the generality of the foregoing, the invention in one preferred embodiment comprises such multi-unit ribozymes which target hybrid oncogenes characteristic of leukemias. Such disorders include, for example, CML and acute lymphocytic leukemia, and other leukemias, such as those involving the c-myc proto-oncogene and bcl-1 and bcl-2. These disorders are characterized by the presence of a tumorigenic chimeric protein resulting from a chromosomal translocation. The multi-unit ribozyme of the invention is designed to cleave the mRNA encoding the tumoregenic protein at a site on the mRNA at or near the translocation junction.

Furthermore, without limiting the generality of the foregoing, the invention is exemplified by a novel RNA molecule designed for cleavage of the bcr-abl mRNA, which mRNA translates the p210$^{bcr-abl}$ protein characteristic of Ph-positive leukemias. However, as noted above, the present invention is not limited to this molecule, but encompasses other molecules designed according to the invention for cleavage of similar genes in other cancers. Ribozymes according to the invention include catalytically active segments for cleaving the selected target chimeric gene transcript after the ribonucleotide sequence G-U-X, wherein X is the ribonucleotide A, U or C.

While the presently preferred ribozyme, as exemplified by Fig. 1, contains three sub-units, ribozymes of four, five, six, seven, eight or more subunits are contemplated. The multi-unit ribozyme targets the oncogene mRNA transcript. Each ribozyme subunit contains a catalytically active segment comprising a ribozyme capable of cleaving oncogene mRNA and

flanking segments having a nucleotide sequence complementary
to portions of the oncogene mRNA. The flanking sequences
serve as "annealing arms" providing specificity of the
ribozyme for the oncogene transcript.

5   It should be noted that absolute complementarity of the
annealing arms to the target oncogene mRNA transcript is not
necessary. For instance, the multi-unit ribozyme may include
restriction enzyme cleavage sites separating the subunits,
to aid in cloning of the intact multiple-unit ribozyme or its
10   separate subunits. Thus, reference herein to an oligonucleo-
tide segment "having a nucleotide sequence complementary to
the nucleotide sequence of a portion of an oncogene mRNA
transcript" does not necessarily mean a sequence having 100%
complementarity with the transcript. In general, the multi-
15   unit ribozyme as a whole will have sufficient complementarity
to the target message to form a duplex therewith of sufficient
stability to allow the catalytically active segments of the
ribozyme to cleave the message at the target GUX sites. Thus,
only sufficient complementarity to permit the ribozyme to be
20   "hybridizable" with the target message is required. One
skilled in the art may readily determine the degree of mis-
matching which may be tolerated, based upon the melting point,
and therefore the stability, of the duplex.

The annealing arms may comprise segments of any length
25   effective is imparting the necessary degree of target
specificity to the ribozyme. Preferably, each annealing arm
comprises from about 4 to about 24 nucleotides, most
preferably from about 6 to about 12 nucleotides.

The triple-unit ribozyme exemplified by Fig. 1 contains
30   three catalytic segments which are positioned so as to cleave
the target bcr-abl mRNA at a GUC triplet 32 nucleotides
upstream (i.e., in the 5'-direction) from the bcr-abl
junction, in the bcr-derived portion of the transcript; at
a GUU triplet 4 nucleotides upstream from the junction; and
35   at a GUA triplet in the abl-derived portion of the transcript,
located 17 nucleotides downstream (i.e., in the 3'-direction)
from the bcr-abl junction. The complete cDNA encoding the
p210$^{bcr-abl}$ protein is disclosed by Schtivelman et al., Nature
315, 550-553 (1985), the entire disclosure of which is

- 10 -

incorporated herein by reference.   As the cDNA sequence
indicates, there are yet additional GUX cleavage sites, dis-
posed even further from the translocation junction, which may
be targeted by a larger multi-unit ribozyme according to the
5   present invention.

Preferably, at least one, and more preferably all, the
catalytic segments comprise the ribonucleotide sequence
CUGAUGAGUC CGUGAGGACG AAA (SEQ ID NO:8). Cleavage of the
target RNA occurs after the GUX sequence wherein X is A, U
10   or C, generating 2'-3' cyclic phosphate and 5'-hydroxyl.
After the target is cleaved, the multi-unit ribozyme is
designed to release the cleaved substrate fragments. The
ability to release the fragments is to some extent dependent
on the length of the flanking sequences. The same multi-unit
15   ribozyme may then cleave other messages and release the
fragments until the molecule is degraded.

The ribozyme of the invention may be prepared by
chemical synthesis or produced in recombinant vectors by
conventional means. See, Molecular Cloning: A Laboratory
20   Manual, Cold Spring Harbor Laboratory (2nd ed., 1989). The
ribozyme RNA sequences may be synthesized conventionally, for
example, by using RNA polymerases such as T7 or SP6.
According to one method exemplified in the examples below,
cDNA encoding the single-unit 23-mer ribozyme SEQ ID NO:1 is
25   synthesized, ligated into multi-unit ribozyme cDNA and cloned
into conventional commercially-available vectors such as
PGEM3z, by conventional techniques. Ribozyme RNA is then
transcribed with an RNA polymerase, e.g., T7 or SP6, by
conventional protocols. The protocols of the commercial
30   suppliers of the cloning vectors and RNA polymerase may be
conveniently followed.

Alternatively, synthetic double-stranded oligode-o-
xyribonucleotide with flanking restriction sites (e.g., BamHI
sites) may be prepared and used to carry out in vitro
35   polymerization (PCR) utilizing Taq polymerase, as described
in WO 93/03141 (1993), incorporated herein by reference. The
PCR product is then cloned into the corresponding restriction
site (e.g., BamHI) of a conventional vector, e.g. pBLUESCRIPT
II KS vector (Stratagene, La Jolla, CA). Transcription of

- 11 -

RNA from the plasmid vector using T7 promotor is carried out,
as described in WO 93/03141.

For therapeutic use, the multi-unit ribozyme may be
combined with a pharmaceutical carrier, such as a suitable
5   liquid vehicle or excipient and an optional auxiliary additive
or additives.   The liquid vehicles and excipient are
conventional and commercially available. Illustrative thereof
are distilled water, physiological saline, aqueous solution
of dextrose, and the like. For in vivo use the ribozyme is
10   preferably administered parenterally, most preferably intra-
venously. The vehicle is designed accordingly. Alternative-
ly, the agent be administered subcutaneously via controlled
release dosage forms.

In addition to administration with conventional
15   carriers, the ribozyme may be administered by a variety of
specialized oligonucleotide delivery techniques. For example,
the ribozyme may be encapsulated in liposomes for therapeutic
delivery, according to conventional oligonucleotide encap-
sulation techniques. The oligonucleotide, depending upon its
20   solubility, may be present both in the aqueous layer and in
the lipidic layer, or in what is generally termed a liposomic
suspension. The hydrophobic layer, generally but not exclu-
sively, comprises phospholipids such as lecithin and sphin-
gomyelin, steroids such as cholesterol, ionic surfactants such
25   as diacetylphosphate, stearylamine, or phosphatidic acid,
and/or other materials of a hydrophobic nature. Oligonucleo-
tides have been successfully encapsulated in unilamellar
liposomes. The liposome interacts spontaneously with DNA or
RNA to form a lipid-DNA or lipid-RNA complex with complete
30   entrapment of the DNA or RNA, and the fusion of this complex
with cell membranes results in efficient uptake of DNA or RNA.
Liposomes formed from cationic materials, e.g., N-[1-(2,3-
dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA),
are preferred.

35   Reconstituted Sendai virus envelopes have been suc-
cessfully used to deliver RNA and DNA to cells. Arad et al.,
Biochem. Biophy. Acta. 859, 88-94 (1986).

The ribozyme may be conjugated to poly(L-lysine) to
increase cell penetration. Such conjugates are described by

Lemaitre et al., Proc. Natl. Acad. Sci. USA, 84, 648-652
(1987). The resulting aldehyde groups are then randomly
coupled to the ε-amino groups of lysine residues of poly(L-
lysine) by Schiff base formation, and then reduced with sodium
5   cyanoborohydride. This procedure converts the 3'-terminal
ribose ring into a morpholine structure.

The ribozyme may be conjugated for therapeutic admini-
stration to cell surface ligand-binding molecules which
recognize cell-surface molecules, such as according to WO
10   91/04753 (1991), the entire disclosure of which is incorpor-
ated herein by reference. The ligand-binding molecule may
comprise, for example, an antibody against a cell surface
antigen, an antibody against a cell surface receptor, a growth
factor having a corresponding cell surface receptor, an
15   antibody to such a growth factor, or an antibody which
recognizes a complex of a growth factor and its receptor.
Methods for conjugating ligand-binding molecules to oligo-
nucleotides are detailed in WO 91/04753.

In particular the growth factor may comprise transferrin
20   or folate. Transferrin-polylysine-oligonucleotide complexes
or folate-polylysine-oligonucleotide complexes may be prepared
for uptake by cells expressing high levels of transferrin or
folate receptor. The preparation of transferrin complexes
as carriers of oligonucleotide uptake into cells is described
25   by Wagner et al., Proc. Natl. Acad. Sci. USA 87, 3410-3414
(1990). The preparation of folate complexes is described in
the examples, below. Inhibition of leukemia cell proliferation
by transferrin receptor-mediated uptake of c-myb antisense
oligonucleotides conjugated to transferrin has been demon-
30   strated by Citro et al., Proc. Natl. Acad. Sci. USA 89, 7031-
7035 (1992). Cellular delivery of folate-macromolecule
conjugates via folate receptor endocytosis, including delivery
of an antisense oligonucleotide, is described by Low et al.,
U.S. Patent 5,108,921. Also see, Leamon et al., Proc. Natl.
35   Acad. Sci. USA 88, 5572 (1991).

As an alternative to treatment with exogenous ribozyme,
the therapeutic agent may be synthesized in situ in the
targeted neoplastic cells with a vector containing an artifi-
cially-constructed gene comprising a transcriptional promotor

- 13 -

and DNA encoding the ribozyme. Upon transcription, the gene
segment transcribes the ribozyme in situ in the targeted cell.
The endogenously produced RNA hybridizes to the relevant mRNA,
resulting in interference with the target gene's function and
5   inhibition of the proliferation of the targeted neoplastic
cell.

The promotor segment of the artificially-constructed
gene serves as a signal-conferring expression of the inserted
DNA which lies downstream thereof. It will include all of
10   the signals necessary for initiating transcription of the
sequence. The promotor may be of any origin as long as it
specifies a rate of transcription which will produce
sufficient quantities of the ribozyme to inhibit the expres-
sion of the target oncogene, and therefore the proliferation
15   of the tumorigenic cells expressing that gene. Preferably,
a highly efficient promotor such as a viral promotor is em-
ployed. Other sources of potent promotors include cellular
genes that are expressed at high levels. The promotor segment
may comprise a constitutive or a regulatable promotor. A
20   typical construct will utilize the SV40 promotor or Maloney
murine leukemia virus long terminal repeat (LTR) sequences.

The artificial gene may be introduced by any of the
methods described in U.S. Patent 4,740,463, incorporated
herein by reference. One technique is transfection, which
25   can be performed by several different methods. One method
of transfection involves the addition of DEAE-dextran to
increase the uptake of the naked DNA molecules by a recipient
cell. See McCutchin, J.H. and Pagano, J.S., J. Natl. Cancer
Inst. 41, 351-7 (1968). Another method of transfection is
30   the calcium phosphate precipitation technique which depends
upon the addition of Ca^{++} to a phosphate-containing DNA
solution. The resulting precipitate apparently includes DNA
in association with calcium phosphate crystals. These
crystals settle onto a cell monolayer; the resulting
35   apposition of crystals and cell surface appears to lead to
uptake of the DNA. A small proportion of the DNA taken up
becomes expressed in a transfectant, as well as in its clonal
descendants. See Graham, F.L. and van der Eb, A.J., Virology
52, 456-467 (1973) and Virology 54, 536-539 (1973).

- 14 -

Transfection may also be carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). See Felgner et al.,

5   Proc. Natl. Acad. Sci. USA 84, 7413-7417 (1987) (DNA-transfection); Malone et al., Proc. Natl. Acad. Sci. USA, 86, 6077-6081 (1989) (RNA-transfection); Zhu et al., Science 261, 209-211 (1993). Polylysine-folate mediated transfection, as described elsewhere herein, may also be used.

10   Alternatively, the artificially-constructed gene can be introduced in to cells, in vitro or in vivo, via a transducing viral vector. See Tabin et al., Mol. Cel. Biol. 2, 426-436 (1982). Use of a retrovirus, for example, will infect a variety of cells and cause the artificial gene to be inserted

15   into the genome of infected cells. Such infection could either be performed with the aid of a helper retrovirus, which would allow the virus to spread through the organism, or the antisense retrovirus could be produced in a helper-free system, such as ψ2-like cells (See Mann et al., Cell 33, 153-160,

20   1983) that package amphotropic viruses. A helper-free virus might be employed to minimize spread throughout the organism. Viral vectors in addition to retroviruses can also be employed, such as papovaviruses, SV40-like viruses, or papilloma viruses. The use of retroviruses for gene transfer has been

25   reviewed by Eglitis and Anderson, BioTechniques 6, 608-614 (1988).

According to one embodiment of retroviral delivery, the multi-unit ribozyme sequence is cloned into a vector containing a transcriptionally active CMV promotor, an SV-40

30   viral promotor, the human β-actin gene promotor, and the T7 polymerase promotor. The constructs may contain a marker, e.g., the gene for neomycin resistance, to allow for selection of useful clones. These DNA constructs are transfected into PA317 fibroblast cells by the calcium-phosphate precipitation

35   method. Cells are plated and grown in the presence of neomycin to select for clones containing DNA coding for ribozymes. These clones are screened for ribozyme RNA using the RNAzol isolation procedure followed by RT-PCR analysis with primers that amplify the ribozyme sequence. NIH-3T3

- 15 -

cells are infected by serial dilution of virus containing
supernatant from the PA317 clones in order to determine the
most efficient ribozyme producing clones. These are selected
for infection of oncogene-expressing cells. Infected cells
5   may be assayed for the chromosomal translocation by detection
of the relevant mRNA transcript using RT-PCR analysis, or by
detection of the tumorgenic protein by immunoprecipi-
tation.   It may be appreciated that in the case of
retroviral delivery of a multi-unti ribozyme specific for bcr-
10   abl, infected cells are assayed for the bcr-abl translocation
by detection of the bcr-abl mRNA transcript or by detecting
the p210$^{bcr-abl}$ protein.

Presently preferred vectors are retroviral vectors,
adenoviral vectors, vaccinia vectors, and others, such as
15   described by E. Gilboa, Adv. Exp. Med. Biol., 241:29 (1988)
and P. H. Pouwels et al., "Vectors for Animal Cells", in
Cloning Vectors: A Laboratory Manual, ch. 7 (Elsevier,
Amsterdam: 1985). Other conventionally employed vectors
designed for use in mammalian, bacterial, yeast, fungal or
20   insect systems may be employed to recombinantly express the
ribozyme of this invention, as exemplified by Fig. 1, but are
not preferred for delivery purposes into the cells.

Vesicle fusion could also be employed to deliver the
artificial gene. Vesicle fusion may be physically targeted
25   to the tumor tissue if the vesicle were appropriately designed
to be taken up by the cells containing the target gene. Such
a delivery system would be expected to have a lower efficiency
of integration and expression of the artificial gene deliv-
ered, but would have a higher specificity than a retroviral
30   vector. A combination strategy of targeted vesicles contain-
ing papilloma virus or retrovirus DNA molecules might provide
a method for increasing the efficiency of expression of tar-
geted molecules.

Still another alternative is to introduce the artificial
35   gene via micro-injection. See for example, Laski et al.,
Cell, 1982.

Particulate systems and polymers for in vitro and in
vivo delivery of polynucleotides was extensively reviewed by
Felgner in Advanced Drug Delivery Reviews 5, 163-187 (1990).

- 16 -

Techniques for direct delivery of purified genes _in vivo_,
without the use of retroviruses, has been reviewed by Felgner
in _Nature_ 349, 351-352 (1991).   Such methods of direct
delivery of polynucleotides may be utilized for local delivery
5   of either exogenous ribozyme or artificially-constructed genes
producing the ribozyme _in situ_.

Recently, Wolf _et al_. demonstrated that direct injection
of non-replicating gene sequences in a non-viral vehicle is
possible. See _Science_, 247, 1465-1468 (1990). DNA injected
10   directly into mouse muscle did not integrate into the host
genome, and plasmid essentially identical to the starting
material was recovered from the muscle months after injection.
Interestingly, no special delivery system is required. Simple
saline or sucrose solutions are sufficient to delivery DNA
15   and RNA.

Preferably, treatment of leukemias, including treatment
of Ph-positive leukemias, is accomplished by contacting bone
marrow cells extracted from a patient _ex vivo_ with a
sufficient number of ribozymes, or vectors carrying ribozymes,
20   for a time sufficient to effect cleavage of the oncogene
present in the cells. Alternatively, the method may employ
contacting the cells _in vivo_, for example, by administration
directly into the bone marrow of a leukemic patient of vectors
carrying the RNA molecule of the invention.   When either
25   method is applied to the cells of CML patients, the RNA
molecule of the invention, as exemplified by Fig. 1, causes
the specific destruction of the bcr-abl mRNA, resulting in
the loss of synthesis of the tumorigenic p210$^{bcr-abl}$ protein.

30   Once transmitted into the cellular environment via a
vector, the ribozyme is expected to survive within the cell
for a short period of time. Since the relationship between
the ribozyme and the target oncogene is not necessarily one-
to-one, a single ribozyme is expected to bind and cleave a
35   number of oncogenic transcripts before being degraded or de-
stroyed by the natural enzymes in the cells and/or the natural
functions of the immune system.

High dose chemotherapy coupled with autologous bone
marrow rescue involves removing a portion of the patient's

- 17 -

bone marrow, treating the patient with conventional chemotherapy or radiation to substantially destroy the remaining bone marrow cells carrying the hybrid oncogene, treating the stored bone marrow with a ribozyme specific for
5   the mRNA transcript of the oncogene, and returning the treated cells to the patient. The treated cells, when returned to the patient, may be stimulated by various known hematopoietic growth factors to repopulate the bone marrow with cells which do not carry the oncogenic transcript. According to a
10   method for bone marrow purging, bone marrow is harvested from a donor by standard operating room procedures from the iliac bones of the donor. Methods of aspirating bone marrow from donors are well-known in the art. Examples of apparatus and processes for aspirating bone marrow from donors are disclosed
15   in U.S. Patents 4,481,946 and 4,486,188, incorporated herein by reference. Sufficient marrow is withdrawn so that the recipient, who is either the donor (autologous transplant) or another individual (allogeneic transplant), may receive from about 4×10^8 to about 8×10^8 processed marrow cells per
20   kg of bodyweight. This generally requires aspiration of about 750 to about 1000 ml of marrow. The aspirated marrow is filtered until a single cell suspension, known to those skilled in the art as a "buffy coat" preparation, is obtained. This suspension of leukocytes is treated with ribozyme in a
25   suitable carrier, advantageously in a concentration of about 50-100 μg/ml. Alternatively, the leucocyte suspension may be stored in liquid nitrogen using standard procedures known to those skilled in the art until purging is carried out. The purged marrow can be stored frozen in liquid nitrogen
30   until ready for use. Methods of freezing bone marrow and biological substances are disclosed, for example, in U.S. Patents 4,107,937 and 4,117,881.

Other methods of preparing bone marrow for treatment with ribozymes may be utilized, which methods may result in
35   even more purified preparations of hematopoietic cells than the aforesaid buffy coat preparation.

One or more hematopoietic growth factors may be added to the aspirated marrow or buffy coat preparation to stimulate growth of hematopoietic neoplasms, and thereby increase their

- 18 -

sensitivity to the toxicity of the ribozyme. Such hemato-
poietic growth factors include, for example, IL-3 and
granulocyte macrophage colony stimulating factor (GM-CSF).
The recombinant human versions of such growth factors are
5   advantageously employed.

       After treatment with the ribozyme, the cells to-be-
transferred are washed with autologous plasma or buffer to
remove unincorporated drug. The washed cells are then infused
back into the patient. Other methods for bone marrow purging
10   utilizing antisense oligonucleotide are disclosed in U.S.
Patent 5,087,617, incorporated herein by reference.

       According to a preferred treatment regimen for bone
marrow purging, the aspirated bone marrow is contacted daily
or twice daily for approximately one to four days with an
15   amount of ribozyme effective to overcome the malignant
phenotype.

       For in vivo administration, the amount of ribozyme may
vary depending on the extent of the neoplasm, the particular
ribozyme utilized, and other factors. The actual dosage
20   administered may take into account the size and weight of the
patient, whether the nature of the treatment is prophylactic
or therapeutic in nature, the age, health and sex of the
patient, the route of administration, whether the treatment
is regional or systemic, and other factors.
25   Sufficient ribozyme should be administered to achieve
an intracellular concentration of from about 1 to about 100
μg/ml. The patient should receive a sufficient daily dosage
to achieve these concentrations of drug. The daily dosage may
range from about 0.1 to 1,000 mg oligonucleotide per day,
30   preferably from about 100 to about 700 mg per day. Greater
or lesser amounts of the oligonucleotide may be administered,
as required. Those skilled in the art should be readily able
to derive appropriate dosages and schedules of administration
to suit the specific circumstance and needs of the patient.
35   It is contemplated that a course of treatment may advan-
tageously comprise infusion of the recommended daily dose of
ribozyme for a period of from about 3 to about 28 days, more
preferably from about 7 to about 10 days. Those skilled in

- 19 -

the art should readily be able to determine the optimal dosage in each case.

For other antisense oligonucleotide drugs, it has been found that a daily oligonucleotide dosage of 250-300 mg will
5   provide an extracellular oligonucleotide concentration of 1.5-2.5 μM which, based upon in vitro studies, has been established as an effective concentration. Thus, for an about 70 kg adult human being, a daily dose of about 250-350 mg oligonucleotide is believed sufficient to achieve an effective
10   extracellular concentration. For children, the daily dosage is reduced proportionately according to the weight of the patient.

The therapeutic end point for either in vivo or ex vivo treatment may be assessed by the absence of cells expressing
15   the relevant chromosomal translocation (e.g., bcr-abl). Recent methods for detecting leukemic cells have focused on detecting the presence of the oncogene, or its corresponding mRNA, in cells of the bone marrow. See, for example, the following U.S. Patents, incorporated herein by reference:
20   4,681,840, 4,857,466 and 4,874,853. Also see Kawasaki et al., Proc. Natl. Acad. Sci. USA 85, 5698-5702 (1988). The presence of even a few copies of the target oncogene can be effectively detected by amplification using reverse transcriptase polymerase chain reaction (RT-PCR) technology.
25   The practice of the invention is illustrated by the following non-limiting examples.

Example 1
Synthesis of Multi-unit Ribozyme
30   cDNA encoding the single-unit 23-mer ribozyme CUGAUGAGUC CGUGAGGACG AAA (SEQ ID NO:1) was synthesized. The single-unit ribozyme cDNA was ligated into double and triple unit ribozymes and cloned into PGEM3z vector (Promega, Madison, WI) (1 μl DNA, 1 μl 10X buffer, 7 μl water, 1 μl low-
35   concentration T4 ligase at 1 U/μl, followed by incubation overnight at 15°C). Ribozyme RNA was transcribed using SP6 RNA polymerase in vitro according to the manufacturer's (Promega) protocol (4 μl 5X TSC buffer; 100 nM DTT; 0.5 μL RNase inhibitor; 1 μg template DNA; 4 μl 1:1:1:1 mixture of

- 20 -

NTPs - U, A, G and C; 1 μl T7 or SP6 RNA polymerase; incubate
at 37°C for 1 hr, then purify product by cold ETOH precipita-
tion). The various ribozymes targeting sites #1, #2 and/or
#3 of the <u>bcr-abl</u> mRNA shown in Fig. 1 were as follows:

          (A)   SEQ ID NO:7 - single-unit ribozyme
targeting site #2
          (B)   SEQ ID NO:2 - single-unit ribozyme
targeting site #1
          (C)   SEQ ID NO:3 - single-unit ribozyme
targeting site #2
          (D)   SEQ ID NO:4 - single-unit ribozyme
targeting site #3
          (E)   SEQ ID NO:5 - double-unit ribozyme
targeting sites #1,2
          (F)   SEQ ID NO:6 - double-unit ribozyme
targeting sites #2,3
          (G)   SEQ ID NO:1 - triple-unit ribozyme
targeting sites #1,2,3

     The two different two-subunit ribozymes targeting site
#2 differed in the length of the annealing arms. Ribozyme
A had shorter annealing arms of 9 nucleotides each, while
ribozyme C had arms of 14 (3') and 16 (5') nucleotides.
     Antisense control ribozymes, with a G substituted for
the A in the conserved GAG sequence in the catalytic unit
eliminating catalytic function, were similarly cloned and
transcribed. Sense ribozymes were synthesized by
transcription from the opposite strand in PGEM3z vector using
T7 RNA polymerase.

Example 2
Cleavage of Target mRNA With Multi-Unit Ribozyme

A. <u>Cleavage of bcr-abl mRNA</u>.

     cDNA encoding a 499 base pair segment of the <u>bcr-abl</u>
breakpoint region was cloned into PGEM3 vector (Promega).
RNA was transcribed using ^{32}P-UTP and T7 RNA polymerase, as
described above. The resulting ^{32}P-labelled <u>bcr-abl</u> mRNA

containing the breakpoint region was used as a target for single-, double- and triple-unit ribozymes in 2-hour cleavage reactions. Reactions were carried out in 10 μl volumes. All RNA concentrations were 100 nM. RNA was suspended in 50 mM

5   Tris-HCl, pH 7.5, 1 mM EDTA. Samples containing substrate and ribozyme were heated to 95°C for 5 minutes, then placed on ice. Cleavage reactions were initiated by adding 1 ml of 200 mM MgCl. Reactions were stopped after 2 hours by adding 2 μl of formamide-EDTA dye. Cleavage products were analyzed

10   by electrophoresis on a 6% sequencing gel followed by autoradiography and quantitative densitometry (Fujix, Tokyo, Japan).

The results are shown in Fig. 2. Lane S represents a control and contains substrate without ribozymes. The

15   ribozymes generating the fragments in Lanes A through G correspond to ribozymes A through G, respectively. Cleavage with double and triple-unit ribozymes released small fragments which ran at the bottom of the gel and are not shown.

As shown in Fig. 2, the ribozymes specifically cleaved

20   bcr-abl mRNA. After 2 hours, single-unit ribozymes cleaved target bcr-abl mRNA into two fragments with variable efficiency. Ribozymes A, B. C and D cleaved 45%, 79%, 5% and 7% of target mRNA, respectively. Comparing ribozymes A and C demonstrates that ribozymes targeting the same site with

25   shorter annealing arms are somewhat more efficient. Double-unit ribozymes E and F cleaved 72% and 13% of target mRNA. There was no significant improvement in cleavage over the best single-unit ribozyme of the pair. However, ribozyme G, the triple-unit ribozyme, cleaved 94% of target mRNA in 2 hours.

30   There was 0% cleavage of bcr-abl with sense- oriented ribozymes and cleavage-inactive antisense ribozymes (data not shown). In addition, adding all three single-unit ribozymes in one reaction resulted in little additional cleavage compared to the best single ribozyme (data not shown).

35

B.   Cleavage of c-abl and bcr mRNA.

In order to test the specificity of the triple-unit ribozyme, cDNAs encoding a 359 base pair segment of the bcr gene comprising nucleotides 563-873 of the sequence reported

- 22 -

by Heisterkamp et al., Nature 315, 758 (1985), and a 447 base
pair segment of the abl gene comprising nucleotides -39 to
+396, were cloned into PGEM5 and PGEM3z vectors (Promega),
respectively. RNA was transcribed using ^{32}P-UTP and T3 and

5   SP6 RNA polymerases, respectively. The resulting ^{32}P-labelled
c-abl and bcr mRNA containing the breakpoint region was used
as a target for single and triple-unit ribozymes in 2-hour
cleavage reactions, as above. The results are indicated in
Figs. 3A and 3B, showing specific cleavage products obtained

10   from c-abl (Fig. 3A) and bcr (Fig. 3B) mRNA. Fig. 3A shows
the cleavage of c-abl control mRNA (lane S, no ribozymes) in
comparison to cleavage by ribozymes D and G. Ribozyme D
cleaved 3% and ribozyme G cleaved 21% of c-abl mRNA. Fig.
3B shows the cleavage of bcr control mRNA (lane S, no

15   ribozymes) in comparison to cleavage by ribozymes B and G.
Ribozyme B cleaved 83% and ribozyme G cleaved 45% of target
bcr mRNA. Thus, the more efficient triple-unit bcr-abl
cleaves c-abl and bcr mRNAs, but significantly less effective-
ly than bcr-abl mRNA (94% of target cleaved).

20

Example 3
Preparation of Folate-polylysine Vector

A.   Preparation of NHS- or Benzotriazole-Folate.

25   Folic Acid (Sigma Chemical Co., St. Louis, MO, 1 g, 2.1
mmol) was dissolved in 10 ml of dimethyl sulfoxide (Fluka)
pre-dried with a molecular sieve. To this solution, N-
hydroxysuccinimide (NHS) (Pierce, 0.242 g., 2 mmol) or N-
hydroxybenzotriazole (Fluka, 0.270 g., 2 mmol) was added.

30   At this point 1,3-dicyclohexylcarbo-diimide (Fluka, 0.872 g.,
4 mmol) dissolved in a small volume of dimethyl sulfoxide was
then slowly added to this solution. The reaction was allowed
to proceed overnight in the dark at room temperature under
stirring. Dicyclohexylurea formed as a white precipitate was

35   removed by filtering twice the reaction mixture through 40
mesh filter paper. The DMSO was then removed by distillation
under vacuum at 45°C until the solution became viscous gel.
The product (NHS-folate or benzotriazole-folate) was then
crystallized in an excess volume of anhydrous ether. A small

- 23 -

amount of methanol was added to accelerate the crystallization process. A fine yellow powder of NHS-folate or benzotriazole-folate formed at this point was subsequently washed twice with anhydrous ether. The product was then dried under vacuum and stored at 4°C.

B. Preparation of Folate-Polylysine Complexes.

Polylysine (pLYS$_{90}$, Sigma, 40 mg) was dissolved in 50 mM carbonate/bicarbonate buffer at pH 9.5. A ten-fold molar excess of NHS-folate or benzotriazole-folate dissolved in 500 μl of DMSO was slowly added to the polylysine solution. The pH of the reaction mixture was adjusted to 9, and the reaction was allowed to proceed at room temperature for at least one hour. The reaction mixture was centrifuged and the super-natant was collected. The folate-conjugated polylysine was then separated from free folate by gel-filtering using a PD-10 desalting column (Pharmacia).

C. Preparation of Folate-polylysine Vector Containing Ribozyme.

Triple-unit bcr-abl ribozyme (SEQ ID NO:1) and folate-polylysine were suspended in 50 μl of a buffer comprising 150 mM NaCl, 20 mM Hepes, pH 7.3 (using RNAse-free water) were mixed gently for 15 minutes. Folate-lysine conjugates of the triple-subunit ribozyme formed spontaneously.

Example 4
Preparation of Liposome Vector

Lipsosomes containing triple-unit bcr-abl ribozyme (SEQ ID NO:1) were prepared in 100 μl volume as follows. RNA and lipofectin   (N-[1-(2,3-diolcyloxy)propyl]-N,N,N-trimethylammonium chloride, "DOTMA") were combined in 100 μl of RNase-free water and gently mixed for 15 minutes. Liposomes containing the triple-subunit ribozyme formed spontaneously.

- 24 -

Example 5
Ribozyme Transfection of Cells
A. Transformation of 32D Cells

32D cells, a murine bone marrow stem cell line, was
5   maintained in IMDM with 10% Fetal Bovine Serum (FBS) and 10%
WEHI supernatant as a source of murine interleukin-3 (IL-3).
A bcr-abl cDNA was transfected into 32D cells by
electroporation.   Transformed cells were selected for by
growth in IL-3-deficient conditions and by neomycin
10   resistance. The presence of the p210$^{bcr-abl}$ tyrosine kinase was
confirmed by northern blotting and immunoprecipitation, and
autophosphorylation with ^{32}P-ATP in a kinase assay. Trans-
formed cells were maintained in IMDM with 10% FBS.

15   ### B. Multi-unit Ribozyme Transfection of K562 and Transformed 32D Cells.

Transformed 32D cells maintained in IMDM with 10% FBS
and cells of the CML cell line K562 maintained in RPMI with
10% FBS were used in the following transfection studies. All
20   cells were washed twice in phosphate-buffered saline (PBS)
prior to transfection. In liposome transfection, K562 cells
were suspended in reduced serum media (OptimMem 1, Gibco,
Gaithersburg, MD) and transformed 32D cells were suspended
in serum-free IMDM. Folate-polylysine mediated transfections
25   were carried out in serum-free, folate-free DMEM after K562
and transformed 32D cells were grown in this media with 10%
FBS for 3 days.   Folate-polylysine and liposome vectors
containing ribozyme were prepared as in the preceding
Examples.   The transfection mix (triple-unit ribozyme in
30   liposome or in folate-polylysine) was added to 4 ml of media
containing 1 x 10^6 K562 of transformed 32D cells.

After 3 hours of ribozyme exposure, total cellular RNA
was isolated and was analyzed by electrophoresis on a 6%
sequencing gel followed by autoradiography and quantitative
35   densitometry.   Fig. 4A shows the improvement in ribozyme
uptake in K562 cells using liposome vectors (lane 2) versus
naked multi-unit ribozymes (lane 1).

Figs. 4B and 4C compare 32D cell transfection using 1
µg of RNA with increasing quantities of folate-polylysine or

- 25 -

lipofectin vector. Using 1 μg of ^{32}P-labelled ribozyme, cells
were transfected with concentrations of lipofectin or folate-
polylysine that varied over 4 logs. Ribozyme uptake was
greater by folate receptor-mediated transfection. As was
5   demonstrated for liposome vectors, folate-polylysine vectors
protected ribozymes from being degraded in serum-free media
(data not shown).

Example 6

10 Serial Cell Transfection with Ribozyme Folate-polylysine
Vector

        To test the availability of the folate receptor for
serial transfections, 32D cells were serially transfected with
unlabeled and ^{32}P-labeled ribozymes over 48 hours. Three
15 identical transfections were performed at time zero with
either ^{32}P-labeled (one sample) or unlabeled ribozymes (two
samples). After 3 hours, the cells transfected with labeled
ribozymes had RNA isolated. Cells transfected with unlabeled
ribozymes were supplemented with FBS (10%), IL-3 and 10% WEHI
20 supernatant. After 24 hours, one of the two remaining samples
was then washed in PBS and transfected with labeled ribozymes.
After 3 additional hours, these cells were washed, had RNA
isolated and stored. A second transfection with unlabeled
ribozymes was performed on the remaining cells (second dose)
25 and these cells were supplemented as described. After 48
hours, these cells were transfected with labeled ribozymes
(third dose) and after 3 hours had RNA isolated as described
above. The RNA was analyzed by electrophoresis on a 6%
sequencing gel followed by autoradiography and quantitative
30 densitometry. Additional samples were transfected with
unlabeled ribozymes and used to maintain cell numbers at 1
x 10^6 prior to the daily transfection with ribozymes.

        The results in Fig. 5 indicate that, compared to the
quantity of labeled ribozyme recovered from cells at 3 hours,
35 50% was recovered after the second transfection and 60% was
recovered after the third transfection. Thus, significant
quantities of ribozyme continued to be delivered via the
folate receptor by serial transfections.

- 26 -

Example 7
Analysis of Cellular RNA Following Transfection with Ribozyme

The ability of multi-unit ribozyme to cleave bcr-abl
mRNA in cells was determined as follows. Untransformed 32D
cells (1×10^6) were added to 1 to 1×10^4 transformed 32D
cells and transfected 1 - 3 times over 24-48 hours with
ribozymes via liposomes or folate-polylysine vectors, inactive
ribozymes, or transfection media containing no ribozymes.
Duplicate transfections were prepared and treated from 1 to
3 times over 24-48 hours. Three hours after transfection,
the media was supplemented with FBS and IL-3 as described
above. After one or two transfections in 24 hours, or after
three transfections in 48 hours, total cellular RNA was
extracted by lysing washed cells in Ultraspec™ solution
(Biotecx, Houston, TX) according to the manufacturer's
protocol. Reverse transcription and polymerase chain reaction
(RT-PCR) were performed according to Kawasaki et al., PCR
Protocols: A Guide to Methods and Applications, Academic
Press, Inc., New York, NY, (1990), p. 21-27, using primers
specific for the bcr-abl translocation. The primer sequences
were GGAGCTGCAG ATGCTGACCA C (SEQ ID NO:9) and TCAGACCCTG
AGGCTCAAAG TC (SEQ ID NO:10) The β-actin gene was also
amplified as an internal control. Twenty µl of the PCR
product was run on 1.5% agarose gel and photographed. South-
ern blotting was performed as described by Maniatis et al.,
Molecular Cloning: A Laboratory Manual, Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, NY (1982), using a probe
spanning the bcr-abl junction, GCTGAAGGGC TTTTGAACTC TGCTTA
(SEQ ID NO:11).

The results are shown in Fig. 6. Transfection with
triple-unit ribozyme via folate-polylysine vector resulted
in a 3 log decrease in detectable bcr-abl mRNA signal after
one transfection in 24 hours. A smaller effect was seen by
transfection with ribozymes via liposomes. Cleavage inactive
ribozymes had no effect on detection of the bcr-abl mRNA
signal (data not shown). RT-PCR using primers that amplified
the β-actin gene confirmed that similar quantities of RNA were
used in RT-PCR amplification (data not shown).

- 27 -

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

The present invention may be embodied in other specific
5   forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indication the scope of the invention.

- 28 -

SEQUENCE LISTING

(1)   GENERAL INFORMATION:

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Commonwealth System of Higher Education

    (ii)   INVENTORS: Lance H. Leopold; Scott K. Shore;
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    (iii)   TITLE OF INVENTION: MULTI-UNIT RIBOZYME
INHIBITION OF ONCOGENE EXPRESSION

    (iv)   NUMBER OF SEQUENCES: 11

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        (A)   MEDIUM TYPE: Diskette, 3.50 inch, 720 Kb

        (B)   COMPUTER: IBM PS/2

        (C)   OPERATING SYSTEM: MS-DOS

        (D)   SOFTWARE: WordPerfect 5.1

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- 29 -

    (2) INFORMATION FOR SEQ ID NO:1:

        (i) SEQUENCE CHARACTERISTICS:

            (A) LENGTH: 156 Nucleotides

            (B) TYPE: nucleic acid

5   (C) STRANDEDNESS: single stranded

            (D) TOPOLOGY: linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTCGAAAGUC AGAUGCCUGA UGAGUCCGUG AGGACGAAAC UGGCCGCUGA 50

GACGUCAGGG CUUUUGCUGA UGAGUCCGUG AGGACGAAAC UCUGCUUAAC 100

10 CTAGGGUGGC UGAGUGCUGA UGAGUCCGUG AGGACGAAAC GAUGACAUUC 150

CTTAAG 156

    (2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:

15   (A) LENGTH: 52 Nucleotides

            (B) TYPE: nucleic acid

            (C) STRANDEDNESS: single stranded

            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

20 GGUGGCUGAG UGCUGAUGAG UCCGUGAGGA CGAAACGAUG ACAUUCCTTA 50

AG 52

    (2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:

25   (A) LENGTH: 53 Nucleotides

            (B) TYPE: nucleic acid

            (C) STRANDEDNESS: single stranded

            (D) TOPOLOGY: linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:3:

30 ACGUCAGGGC UUUUGCUGAU GAGUCCGUGA GGACGAAACU CUGCUUAACC 50

TAG 53

    (2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:

35   (A) LENGTH: 51 Nucleotides

            (B) TYPE: nucleic acid

            (C) STRANDEDNESS: single stranded

            (D) TOPOLOGY: linear

- 30 -

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTCGAAAGUC AGAUGCCUGA UGAGUCCGUG AGGACGAAAC UGGCCGCUGA 50

G 51

5   (2)   INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:

             (A) LENGTH: 105 Nucleotides

             (B) TYPE: nucleic acid

             (C) STRANDEDNESS: single stranded

10   (D) TOPOLOGY: linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACGUCAGGGC UUUUGCUGAU GAGUCCGUGA GGACGAAACU CUGCUUAACC 50

TAGGGUGGCU GAGUGCUGAU GAGUCCGUGA GGACGAAACG AUGACAUUCC 100

TTAAG 105

15

    (2)   INFORMATION FOR SEQ ID NO:6:

        (i) SEQUENCE CHARACTERISTICS:

             (A) LENGTH: 104 Nucleotides

             (B) TYPE: nucleic acid

20   (C) STRANDEDNESS: single stranded

             (D) TOPOLOGY: linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:6:

 TTCGAAAGUC AGAUGCCUGA UGAGUCCGUG AGGACGAAAC UGGCCGCUGA 50

GACGUCAGGG CUUUUGCUGA UGAGUCCGUG AGGACGAAAC UCUGCUUAAC 100

25 CTAG 104

    (2)   INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:

             (A) LENGTH: 41 Nucleotides

30   (B) TYPE: nucleic acid

             (C) STRANDEDNESS: single stranded

             (D) TOPOLOGY: linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:

35 GGGCUUUUGC UGAUGAGUCC GUGAGGACGA AACUCUGCUU A 41

    (2)   INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:

    (A) **LENGTH:** 23 Nucleotides

    (B) **TYPE:** nucleic acid

    (C) **STRANDEDNESS:** single stranded

    (D) **TOPOLOGY:** linear

5

   (xi) **SEQUENCE DESCRIPTION: SEQ ID NO:8:**

CUGAUGAGUC CGUGAGGACG AAA 23

  (2) **INFORMATION FOR SEQ ID NO:9:**

10   (i) **SEQUENCE CHARACTERISTICS:**

    (A) **LENGTH:** 21 Nucleotides

    (B) **TYPE:** nucleic acid

    (C) **STRANDEDNESS:** single stranded

    (D) **TOPOLOGY:** linear

15   (xi) **SEQUENCE DESCRIPTION: SEQ ID NO:9:**

GGAGCTGCAG ATGCTGACCA C 21

  (2) **INFORMATION FOR SEQ ID NO:10:**

   (i) **SEQUENCE CHARACTERISTICS:**

20   (A) **LENGTH:** 22 Nucleotides

    (B) **TYPE:** nucleic acid

    (C) **STRANDEDNESS:** single stranded

    (D) **TOPOLOGY:** linear

   (xi) **SEQUENCE DESCRIPTION: SEQ ID NO:10:**

25 TCAGACCCTG AGGCTCAAAG TC 22

  (2) **INFORMATION FOR SEQ ID NO:11:**

   (i) **SEQUENCE CHARACTERISTICS:**

    (A) **LENGTH:** 26 Nucleotides

30   (B) **TYPE:** nucleic acid

    (C) **STRANDEDNESS:** single stranded

    (D) **TOPOLOGY:** linear

   (xi) **SEQUENCE DESCRIPTION: SEQ ID NO:11:**

GCTGAAGGGC TTTTGAACTC TGCTTA 26

- 32 -

CLAIMS

1. A synthetic RNA molecule useful for the treatment of a neoplasm characterized by the presence of a hybrid oncogene resulting from a chromosomal translocation comprising

(a) a first ribozyme subunit comprising

(i) a first flanking segment having a nucleotide sequence complementary to the nucleotide sequence of a portion of the oncogene mRNA transcript substantially 5' of the oncogene translocation junction and being hybridizable to that mRNA portion;

(ii) a second flanking segment having a nucleotide sequence complementary to the nucleotide sequence of a portion of the oncogene mRNA transcript substantially 3' of the oncogene translocation junction and being hybridizable to that mRNA portion;

(iii) a catalytically active segment disposed between the first and second flanking segments comprising a ribozyme capable of cleaving the oncogene mRNA at or near the translocation junction; and

(b) two or more additional ribozyme subunits, each subunit comprising a catalytically active segment comprising a ribozyme capable of cleaving the oncogene mRNA and flanking segments complementary to the oncogene mRNA transcript and hybridizable thereto.

2. An RNA molecule according to claim 1 for the treatment of a leukemia.

3. An RNA molecule according to claim 2 wherein the oncogene is bcr-abl, and the RNA molecule is useful for the treatment of a Ph-positive leukemia.

4. An RNA molecule according to claim 1 wherein at least one of the catalytically active segments comprises the nucleotide sequence SEQ ID NO:8.

5.   An RNA molecule according to claim 1 containing one or more restriction enzyme sites for cloning of said molecule or subunit thereof.

6.   An RNA molecule according to claim 1 wherein the flanking segments comprise from about 4 to about 24 nucleotides.

7.   An RNA molecule according to claim 6 wherein the flanking segments comprise from about 6 to about 12 nucleotides.

8. An RNA molecule according to claim 1 comprising three ribozyme subunits.

9.   A recombinant vector comprising DNA which when expressed will provide an RNA molecule according to claim 1.

10.   A pharmaceutical composition comprising an RNA molecule according to claim 1 and a pharmaceutically acceptable carrier.

11.   A composition according to claim 10 wherein the carrier comprises a liposome encapsulating the RNA molecule.

12. A liposome composition according to claim 11 wherein the liposome is formed from a cationic lipid.

13.   A liposome composition according to claim 12 wherein the cationic lipid is N-[1-(2,3-diolcyloxy)propyl]-N,N,N-trimethylammonium chloride.

14. A conjugate comprising an RNA molecule according to claim 1 linked to a cell surface ligand-binding molecule.

15. A conjugate according to claim 14 wherein the cell surface ligand-binding molecule is selected from the group consisting of antibodies against cell surface antigens, antibodies against cell surface receptors, growth factors,

antibodies to growth factors, and antibodies which recognize
a complex of a growth factor and its receptor.

16. A conjugate according to claim 15 wherein the cell
surface ligand-binding molecule comprises folate.

17. A conjugate according to claim 16 wherein the cell
surface ligand-binding molecule is folate-polylysine.

18. A method for treating a patient afflicted with a
neoplasm characterized by the presence of a hybrid oncogene
resulting from a chromosomal translocation, which method
comprises contacting cells of the patient with an effective
amount of the RNA molecule of claim 1 for a time sufficient
to cleave the oncogene mRNA and inhibit the expression of the
oncogene.

19. A method according to claim 18 for the treatment of
a leukemia.

20. A method according to claim 19 for the treatment of
a Ph-positive leukemia.

21. A method according to claim 18 wherein the cells are
contacted with said RNA molecule _in vivo_.

22. A method according to claim 18 wherein the cells are
contacted with said RNA molecule _in vitro_.

23. A method according to claim 19 comprising treating
bone marrow cells aspirated from a leukemia patient with said
synthetic RNA molecule, and returning the thus-treated cells
to the body of the afflicted individual.

24. A method for inhibiting the proliferation of cells
characterized by the presence of a hybrid oncogene resulting
from a chromosomal translocation, which method comprises
introducing into such cells an artificially-constructed gene

which, upon transcription in said cells, produces an RNA
molecule according to claim 1.

25. A method according to claim 24 wherein the artifi-
cially-constructed gene is introduced into said cells by
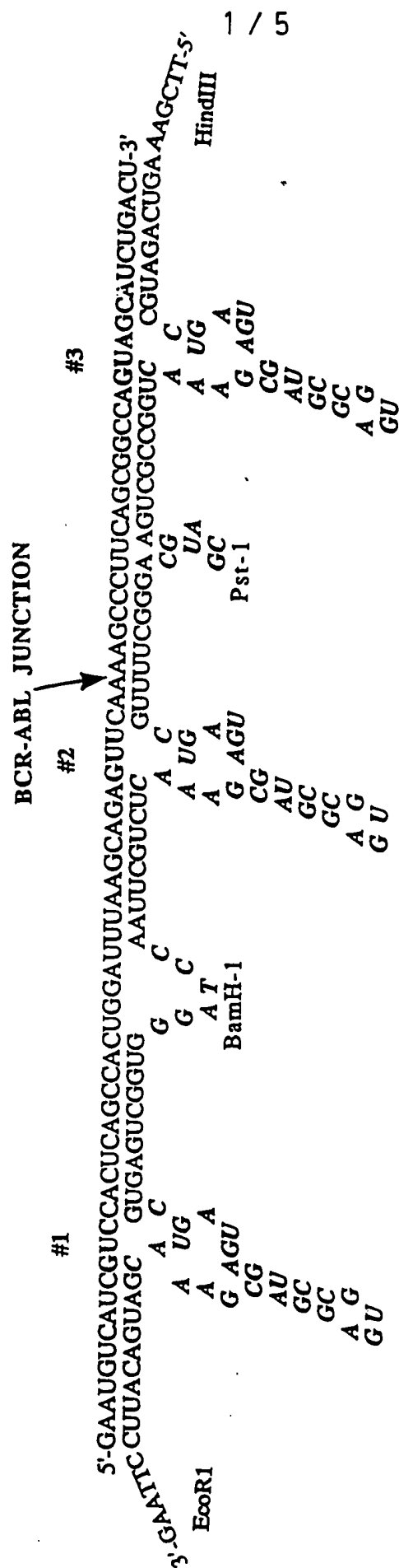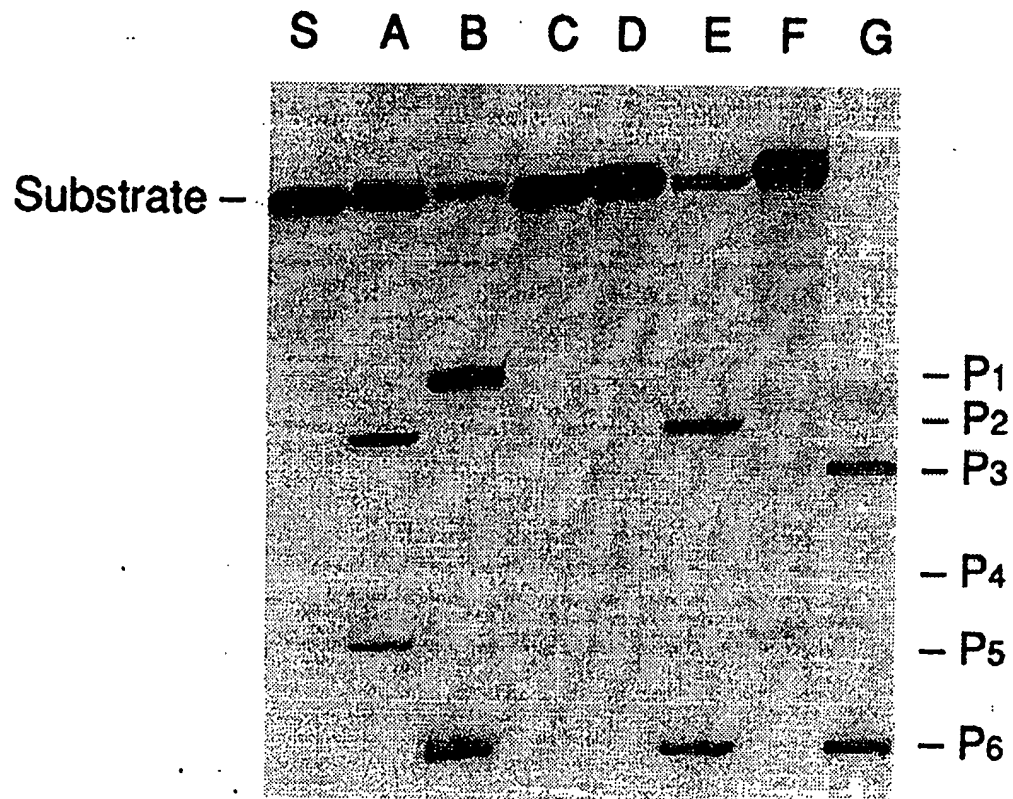transfection, by a transducing viral vector or by
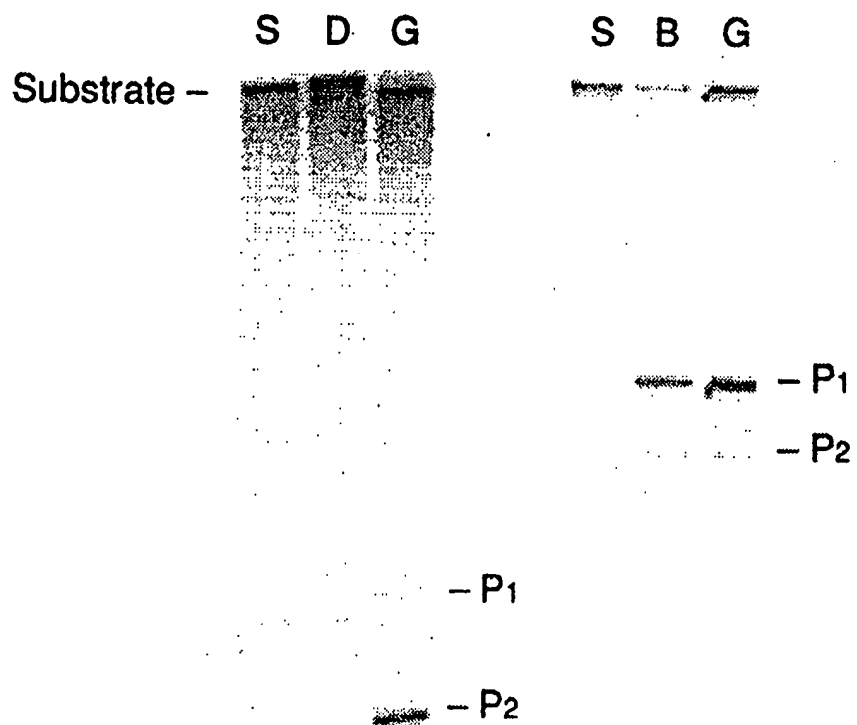microinjection.

FIG. 1

FIG. 2

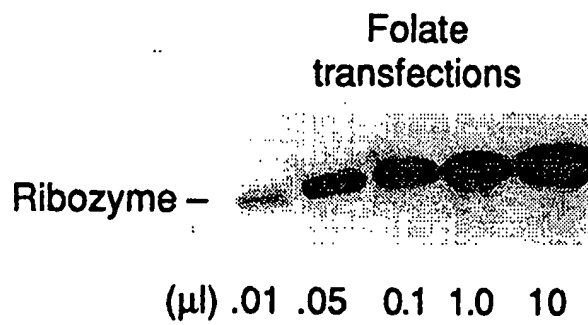FIG. 3A   FIG. 3B

FIG. 4A

Ribozyme –

1   2

Folate
transfections

Liposome
transfections

Ribozyme –

(µl) .01 .05 0.1 1.0 10   .01 .05 0.1 1.0 10 (µg)

FIG. 4B   FIG. 4C

Folate
transfections

Ribozyme —

3   24   48

Hours

FIG. 5

Cell number   +   —   1   10   10² 10³ 10⁴

Control —

Folate
transfections —

Liposome
transfections —
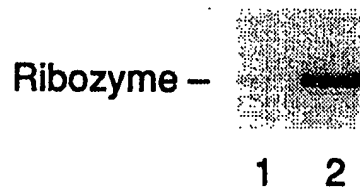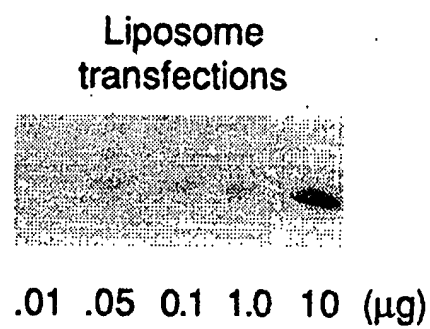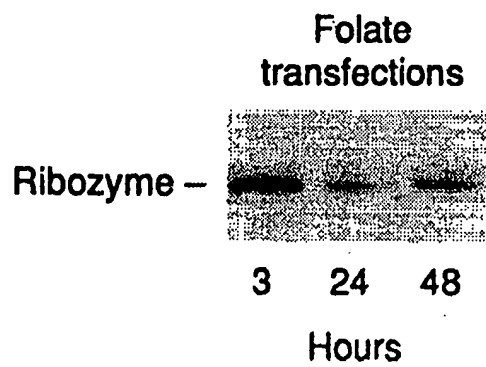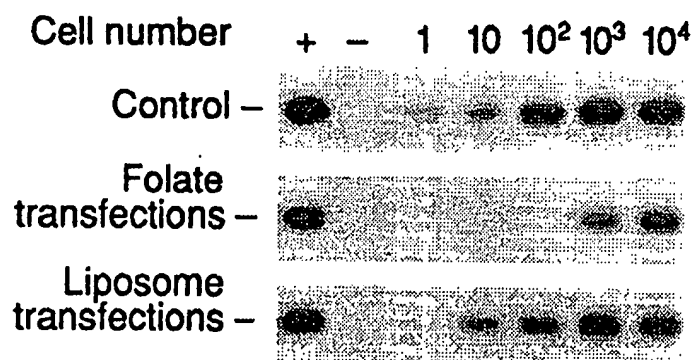
FIG. 6

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C07H 21/02; A61K 48/00, 31/70, 31/74

US CL   :514/44; 536/24.5; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/24.5; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,108,921 (LOW ET AL.) 28 April 1992, col. 6, line 55 to col. 7, line 11, and col. 8, lines 5-14.	14-17
Y	WO, A, 91/04753 (BAER ET AL.) 18 April 1991, see entire document.	14-17
Y	WO, A, 92/00080 (REDDY ET AL.) 09 January 1992, see entire document.	1-25
Y	WO, A, 92/22303 (CALABRETTA ET AL.) 23 December 1992, see entire document.	1-25
Y	WO, A, 93/03141 (SNYDER ET AL.) 18 February 1993, see entire document.	1-25

[X]   Further documents are listed in the continuation of Box C.　　[ ]   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 DECEMBER 1994	11 JAN 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	CHARLES C. P. RORIES, Ph.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation f document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular Pharmacology, Volume 41, issued 1992, C. F. Bennett et al., "Cationic Lipids Enhance Cellular Uptake and Activity of Phosphorothioate Antisense Oligonucleotides", pages 1023-1033, see entire document.	11-13
Y	Proceedings of the National Academy of Sciences USA, Volume 84, issued February 1987, M. Lemaitre et al., "Specific Antiviral Activity of a Poly(L-Lysine)-Conjugated Oligodeoxyribonucleotide Sequence Complementary to Vesicular Stomatitis Virus N Protein mRNA Initiation Site", pages 648-652, see entire document.	17
Y	US, A, 5,087,617 (SMITH) 11 February 1992, col. 15, line 17 to col. 16, line 43.	18-23
Y	Biochemical and Biophysical Research Communications, Volume 147, No. 1, issued 31 August 1987, D. Mercola et al., "Antisense RNA to the c-fos Gene: Restoration of Density-Dependent Growth Arrest in a Transformed Cell Line", pages 288-294, see entire document.	24
Y	Blood, Volume 76, No. 2, issued 15 July 1990, A. D. Miller, "Progress Toward Human Gene Therapy", pages 271-278, see entire document.	25

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, DERWENT WORLD PATENT INDEX
search terms: bcr, abl, ribozyme

A search for the disclosed ribozyme nucleotide sequence in the sequence data banks was not conducted because the CRF contains errors.